DETECTION OF RANTES GENE PROMOTOR POLYMORPHISMS

The present invention relates to methods for the genetic testing of samples to determine the presence of RANTES polymorphisms which are linked to a genetic predisposition to particular diseases or disorders. More particularly, the invention relates to the diagnosis of or detection of a genetic predisposition to protection from infection with the Human Immuno-Deficiency Virus (HIV) and diagnosis of or detection of a predisposition to developing immuno-modulated disorders like asthma.

RANTES (regulated upon activation normal T cell expressed and secreted) belongs to the superfamily of proinflammatory cytokines called "chemokines" and is a member of the chemokine family designated CC chemokines, as the family is characterised by the presence of conserved cysteine (C-C) motifs. RANTES has an important role in the attraction and recruitment of monocytes, memory T cells, basophils and natural killer cells to sites of inflammation (Schall *et al.*, (1990), Nature, 347, page 669, Kameyoshi *et al.*, (1992), J. Experimental Medicine, 176, page 587). RANTES can also cause degranulation of basophils, respiratory burst in eosinophils and stimulation of T cell proliferation. It has anti-viral activity as it has been shown to suppress HIV replication *in vitro* (Schmidtmayerova *et al.*, (1996), Nature, 382, page 767).

In vivo, RANTES is expressed in diseases characterised by a mononuclear cell infiltration such as renal allograft rejection, delayed type hypersensitivity and chronic inflammatory lung disease. This suggests that RANTES plays a role in both acute and chronic inflammation. Nelson *et al.*, (1993), J. Immunol. Vol. 151, pages 2601-2612) suggests that understanding the transcriptional control of RANTES expression should provide new insights into the regulation of inflammation and may give rise to novel immunotherapeutic approaches.

The RANTES gene is located on the long arm of chromosome 17 at 17Q 11.2-q12, together with the other members of the CC chemokine family. The RANTES gene

spans approximately 7.1 kb and is composed of three exons and two introns. The RANTES protein comprises 91 amino acids, including a 23 residue leader sequence. The mature protein is basic, with a molecular weight of 7847 Daltons. Approximately 1 kb of DNA upstream of the transcription start site of the RANTES gene (the promoter region) has been sequenced (Nelson *et al., supra)*. This region was found to contain a large number of potential consensus regulatory elements.

Genetic testing may be defined as the analytical testing of a patient's nucleic acid to determine if the DNA of a patient contains mutations (or polymorphisms) that either cause or increase susceptibility to a disease state or disorder or are in "linkage" with the gene causing a disease state and are thus potentially indicative of a predisposition to the disease state or disorder.

The early detection of a predisposition to a disease or disorder presents the best opportunity for medical intervention. Early genetic identification of risk may improve the prognosis for a patient through early intervention before clinical symptoms of the disease or disorder manifest.

In cases where patients with similar symptoms are treated with variable success with the same therapeutics, genetic testing may differentiate patients with a genetic rather than developmental basis for their symptoms, thus leading to the potential need for different approaches to therapy.

It is an aim of the present invention to provide methods for genetic screening to indicate a risk or predisposition to diseases or disorders.

According to a first aspect of the present invention there is provided an *in vitro* method for diagnosing or detecting a predisposition to a disease or disorder associated with abnormal RANTES gene expression, the method comprising examining the RANTES gene promoter to detect the presence of a genetic polymorphism.

The invention allows an investigator to identifying patients expressing a genetic polymorphism associated with diseases or disorders associated with abnormal RANTES gene expression or a predisposition to such diseases or disorders to determine those patients who have or are more at risk of developing a RANTES associated disease or disorder by solely examining a sample for the presence of a RANTES gene polymorphism. This allows for appropriate action to be taken to prevent or lessen the likelihood of onset of the disease or disorder or to allow appropriate treatment of the disease or disorder.

According to a second aspect of the present invention there is provided a method of treatment for individuals who either have or are predisposed to diseases or disorders associated with abnormal RANTES gene expression by administering to individuals who have a RANTES promoter polymorphism, as determined by the method according to the first aspect of the invention, a modulator of RANTES activity.

The invention is based on the discovery of a particular genetic polymorphism pattern for the RANTES (regulated upon activation normal T cell expressed and secreted) gene that was found to be associated with certain diseases or disorders in which the wild type RANTES gene plays a role. Whilst not wishing to be bound by theory, the inventors believe that particular RANTES polymorphisms may be indicative of a predisposition to abnormal RANTES gene expression, especially up-regulated RANTES gene expression, thereby indicating a predisposition to abnormal amount of RANTES being produced, leading to a disease state.

The term "polymorphism" refers to a different gene sequence from the wild type. Polymorphisms can be variants which are generally found between individuals of different ethnic backgrounds or from different geographical areas, those polymorphisms not affecting the function of the gene. Other polymorphisms are those which lead to differences in the function of the gene or may produce an inactive gene product or may modulate the production of the gene product.

A particular polymorphism associated with diseases or disorders associated with the RANTES gene has been identified in the 5' upstream regulatory region (promoter region) of the RANTES gene. Particularly, a polymorphism of at least one copy of a point mutation of a Guanine nucleotide (G) to an Adenine nucleotide (A) at position –400 relative to the transcription start site of the RANTES gene (based on the Nelson *et al.* sequence) may provide or diagnosis or indicate an increased susceptibility to the diseases or disorders listed below. A polymorphism of at least one copy of a point mutation of a Cytosine (C) to a Guanine (G) at position –28 relative to the transcription start site of the RANTES gene (based on the Nelson *et al.* sequence) may provide or diagnosis or indicate an increased susceptibility to the diseases or disorders listed below.

As outlined above preferred positions on the RANTES promoter for examination for polymorphisms are positions –28 and –400 in relation to the transcriptional start site according to the sequence published in Nelson *et al.*, *supra*. It has come to light that the sequence published in Nelson *et al.*, *supra* is incorrect and a new sequence for RANTES has been disclosed (Lui *et al.*, Proc. Natl. Acad. Sci. USA). The new Lui sequence for RANTES contains some insertions relative to the Nelson sequence and this has resulted in a renumbering of the site of the –400 polymorphism to –403. The site of the –28 polymorphism remains unchanged.

Figure 1 shows the Nelson *et al.* sequence.

Figure 2 shows the Lui *et al.* sequence.

Figure 3 shows an alignment of the Nelson sequence of Figure 1 with the Lui sequence of Figure 2.

The figures clearly show that the –400 position of the promoter as referred to in relation to the Nelson sequence is equivalent to the –403 position of the promoter as disclosed in the Lui paper. Accordingly, all references to the –400 polymorphism in relation to the Nelson paper as described herein are clearly equivalent to a polymorphism at –403 when considered in relation to the sequence disclosed in the Lui paper. Therefore all references to the –400 position of the RANTES promoter (of the Nelson paper) in disclosed herein are to be understood as reference to the –403 position of the RANTES promoter of the Lui sequence.

Particularly the method according to the present invention may be used for diagnosing or detecting a predisposition to a disease or disorder normally associated with increased RANTES production.

RANTES is a potent chemoattractant for memory T cells, natural killer cells, monocytes, eosinophils and basophils. RANTES is also known as causing degranulation of basophils, respiratory burst in eosinophils and stimulation of T cell proliferation. Because of its chemoattractant properties RANTES potentially plays a role in immune regulatory function, as well in auto-immune disorders and inflammatory processes. RANTES is implicated *in vivo* in renal allograft rejection, delayed type hypersensitivity and inflammatory lung disease suggesting a role for RANTES in both acute and chronic inflammatory disorders. RANTES is also implicated as an important factor in the suppression of HIV infection by its role in competing with the virus in infiltrating T cells.

Accordingly, the existence of a RANTES polymorphism in a sample may be indicative of the presence of, or a predisposition to, diseases or disorders associated with abnormal immune regulatory function, auto-immune disorders and inflammatory disorders such as asthma, atopy, allergies, rheumatoid arthritis, oral lichen planus, kidney allograft rejection, heart allograft rejection, heart-lung allograft rejection, insulin dependent diabetes mellitus, auto-immune thyroiditis (Graves disease and

Hashimoto's thyroiditis) psoriasis, vasculitis, giant cell arthritis, acquired immuno-deficiency syndrome (AIDS) as a predictor of protection against and susceptibility to HIV infection, inflammatory bowel disease, multiple sclerosis, dermatomyositis and polymyositis.

The method of the present invention allows patients with or without symptoms for a particular disease or disorder to be identified as having a genetic predisposition to a disease or disorder by detecting the presence of a polymorphism in the RANTES gene.

The method according to the present invention is particularly suitable for detecting those patients with, or having a predisposition to developing, asthma, particularly atopic asthma. Due to the involvement of RANTES with prevention of HIV infection, the method according to the present invention may also be used to indicate those individuals who may have some degree of protection from HIV infection and are therefore less susceptible to developing AIDS.

The *in vitro* test is particularly suitable for being carried out on genomic DNA, particularly on isolated genomic DNA. Such genomic DNA may be isolated from blood or tissue samples or from other suitable sources.

Techniques for determining the presence of a RANTES genetic polymorphism may be nucleic acid techniques based on size or sequence, such as hybridisation techniques, nucleic acid sequencing or restriction fragment length polymorphism (RFLP). These techniques may also involve amplifying the nucleic acid before analysis. Suitable amplification techniques include cloning and polymerase chain reaction (PCR). Amplification products may be assayed in a variety of ways, including detecting specific tagged oligonucleotide primers in the reaction products, size analysis, restriction digestion followed by size analysis, allele-specific oligonucleotide hybridisation and sequencing.

According to the present invention the determination of the existence of determining a RANTES polymorphism may involve amplification by polymerase chain reaction (PCR) of at least a fragment of the DNA.

In a preferred method of the present invention, the sample DNA is subjected to PCR amplification using PCR primers specific for the region around the polymorphic fragment of DNA only. Preferably a region of under 200 bases is amplified from the RANTES promoter using suitable PCR primers. The amplification product may then be analysed by restriction digestion and size analysis. In the situation that neither the wild type nor the mutant allele abolishes or introduces a restriction enzyme site a restriction enzyme site is introduced by the PCR primers into the amplified product. The introduced enzyme site allows differentiation between polymorphic alleles and wild type by size analysis. For example if the restriction products of the amplified product are analysed by gel electrophoresis (SDS polyacrylamide gel, for example) the alleles with the introduced restriction enzyme site produce an extra band on the gel.

PCR primers suitable for amplifying a region around the –400 polymorphism (Nelson sequence, -403 polymorphism Lui sequence) are listed below as SEQ ID No. 1 and SEQ ID No. 2.

Forward primer: 5' GCC TCA ATT TAC AGT GTG 3' (SEQ ID No. 1)

Reverse primer: 5' TGC TTA TTC ATT ACA GAT GTT 3' (SEQ ID No. 2)

The position of the forward primer starts at –513 and the position of the reverse primer is at –378. The PCR product length generated using this primer pair is 135 bp.

So as to create an enzyme site that can be used to differentiate between wild type/polymorphism, a single base change may be introduced into the PCR product by one of the primers, so as to give a different number of restriction enzyme sites between

the wild type and polymorphic sequences. According to a preferred embodiment using the above PCR primer sequences the reverse primer SEQ ID No. 2 has a G base (underlined) at the 3' end of the reverse primer SEQ ID No. 2 mutated from the wild type C base. This is equivalent to a C to G mutation in the wild type at –396 relative to the transcription start site. Such a mutation introduced a restriction enzyme site into the wild type sequence.

After PCR has been performed using the above primers, restriction digestion with the restriction enzyme *MaeIII* in the presence of the wild type cuts the PCR product into two bands of 112 and 23 bp. If the polymorphism is present the enzyme does not cut the PCR product as their presence of the polymorphism mutates the sequence at the restriction enzyme site, therefore only one band, of 135 bp, is produced.

PCR primers suitable for amplifying a region around the –28 polymorphism are listed below as SEQ ID No. 3 and SEQ ID No. 4.

Forward primer: 5' ACA GAG ACT CGA ATT TCC GGA 3' (SEQ ID No. 3)

Reverse primer: 5' CCA CGT GCT GTC TTG ATC CTC 3' (SEQ ID No. 4)

The position of the forward primer starts at –145 and the position of the reverse primer is at +28. The PCR product length generated using this primer pair is 178 bp.

The G polymorphism at position –28 in the human RANTES promoter abolishes *a Mnl I* restriction enzyme cleavage site. In the presence of the wild type C allele the *Mnl I* enzyme digestion of the PCR product yields three bands of 126, 27 and 20 bp. In the presence of the G polymorphism only two bands of 146 and 27 bp are detected.

PCR primers specific for a target sequence of sample DNA potentially containing a polymorphism may be provided in a kit. Suitable PCR primers for the kit include SEQ

ID No. 1 and SEQ ID No. 2 or SEQ ID No. 3 and SEQ ID No. 4 as described above. The kit may also comprise a suitable restriction enzyme, for example the restriction enzyme *MaeIII* is suitable for detecting a polymorphism when used in conjunction with PCR primers SEQ ID No. 1 and SEQ ID No. 2 and the restriction enzyme *Mnl I* is suitable for detecting a polymorphism when used in conjunction with PCR primers SEQ ID No. 3 and SEQ ID No. 4.

Using the method according to the present invention an individual having a polymorphism in the RANTES gene promoter may be identified. This provides a clinician with information of the likelihood of a patient of developing or having a particular disease or disorder and may be important in the clinical management of specific diseases or disorders associated with abnormal RANTES gene expression.

The present invention in a second aspect further provides a method for treating individuals who either have or are predisposed to diseases or disorders associated with abnormal RANTES gene expression by administering to individuals who have a RANTES promoter polymorphism, as determined by the method according to the first aspect of the invention, a modulator of RANTES activity.

Several classes of compound are capable of modulating RANTES activity. For instance, preferred modulators of RANTES activity enhance receptor activity such as: RANTES agonists or partial agonists, agents which enhance the release of endogenous activators of RANTES, agents which enhance the synthesis of endogenous activators of RANTES, agents which attenuate the breakdown of endogenous activators of RANTES and agents which increase RANTES expression or activity.

Preferred compounds that enhance RANTES activity, which may be used according to the invention, are RANTES agonists. For example additional RANTES or active RANTES analogues may be administered to increase the amount of active RANTES.

Whilst compounds which enhance RANTES activity may be useful for treating conditions associated with abnormal RANTES gene expression, we have also found that other types of compound which modulate RANTES activity are effective for controlling such diseases or disorders. For instance, RANTES antagonists, inverse agonists or partial agonists may be used to modulate RANTES activity.

Preferred antagonists for use according to the second aspect of the invention include RANTES(9-68) (see Arenzana-Seisdedos *et al.*, 1996, Nature Vol. 383), and Met-RANTES (see Elsner *et al.*, 1997, Eur J. Immunol. 27(11) pages 2892-8). Other suitable antagonists may include the distamycin analogue 2,2'[4,4'-[[aminocarbonyl] amino]bis[N,4'-di[pyrrole-2-carboxamide-1,1'-dimethyl]]-6,8 napthalenedisulfonic acid] hexasodium salt (NSC 651016), (see Howard *et al.*, 1998, J. Leukoc. Biol. 64(1), pages 6-13), 4-hydroxypiperidine compounds such as (2-2-diphenyl-5-(4-chlorophenyl)piperidin-1-yl)valeronitrite) (see Hesselgesser *et al.*, 1998, J. Biol. Chem. 19;273(25) pages 15687-92) and AOP-RANTES (see Simmons *et al.*, 1997, Science 11;276(5310) pages 276-9.

The compounds may be used to treat existing conditions but may also be used when prophylactic treatment is considered medically necessary.

Treatment of conditions associated with abnormal RANTES gene expression with compounds according to the invention may be either as a monotherapy or in combination with other therapeutic agents.

The modulators of RANTES activity used according to the second aspect of the invention may take a number of different forms depending, in particular on the manner in which the composition is to be used. Thus, for example, the composition may be in the form of a powder, tablet, capsule, liquid, ointment, cream, gel, hydrogel, aerosol, spray, micelle, liposome or any other suitable form that may be administered to a person or animal. It will be appreciated that the vehicle of the

composition of the invention should be one which is well tolerated by the subject to whom it is given and enables delivery of the compounds to the site of action.

Compositions that are RANTES modulators may be used in a number of ways. For instance, systemic administration may be required in which case a suitable compound may be contained within a composition which may, for example, be ingested orally in the form of a tablet, capsule or liquid. Alternatively the composition may be administered by injection into the blood stream. Injections may be intravenous (bolus or infusion) or subcutaneous (bolus or infusion).

The RANTES modulator may also be incorporated within a slow or delayed release device. Such devices may, for example, be inserted under the skin and the compound which modulates RANTES activity may be released over weeks or even months. Such a device may be particularly useful for patients with a long term disorder such as asthma. The devices may be particularly advantageous when a compound is used which would normally require frequent administration (e.g. at least daily ingestion of a tablet or daily injection).

It will be appreciated that the amount of a compound required is determined by biological activity and bioavailability which in turn depends on the mode of administration, the physicochemical properties of the compound employed and whether the compound is being used as a monotherapy or in a combined therapy. The frequency of administration will also be influenced by the above mentioned factors and particularly the half-life of the compound within the subject being treated.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials etc), may be used to establish specific formulations of compositions and precise therapeutic regimes (such as daily doses of the compounds and the frequency of administration).

Generally, a daily dose of between 0.01μg/kg of body weight and 1.0g/kg of body weight of a compound which modulates RANTES activity may be used depending upon which specific compound is used and the condition to be treated. Preferably the daily dose is between 0.01mg/kg of body weight and 100mg/kg of body weight.

Daily doses may be given as a single administration (e.g. a daily tablet for oral consumption or as a single daily injection). Alternatively the compound used may require administration twice or more times during a day. A patient receiving treatment may take a first dose upon waking and then a second dose in the evening (if on a two dose regime) or at 3 or 4 hourly intervals thereafter. Alternatively a slow release device may be used to provide optimal doses to a patient without the need to administer repeated doses.

The diagnosis of a polymorphism in the RANTES gene allows patients who would normally have been treated in one way to be treated with a RANTES modulator, thus avoiding unnecessary treatment with sometimes expensive or harmful drugs, for example steroids. For examples some patients may suffer with asthma because of environmental and developmental pressures. Other patients may suffer from or have a predisposition to developing asthma because of abnormal RANTES gene expression. Using the method of the present invention to determine those patients with a RANTES polymorphism allows these patients to be treated differently from those patients, who whilst displaying similar symptoms, do not have the polymorphism.

Furthermore, the method of the present invention allows identification of those at risk for a disease or disorder so as to allow preventative measured to be initiated prior to disease onset. Further, those patients who have two or more risk factors, the genetic predisposition as indicated by the RANTES polymorphism and life-style predispositions or other known genetic predispositions can be particularly monitored and prophylactically treated since their risk of disease is unusually high.

The invention will be further described, by way of example only, with reference to the accompanying drawing, in which:

Figure 1 shows the Nelson *et al*. sequence.

Figure 2 shows the Lui *et al*. sequence.

Figure 3 shows an alignment of the Nelson sequence of Figure 1 with the Lui sequence of Figure 2.

Figure 4 shows an agarose gel stained with ethidium bromide to illustrates the results of restriction digestion with *MaeIII* of PCR products generated using RANTES PCR primers.

Figure 5 shows a table of polymorphisms found in asthmatic and HIV exposed patients, and controls.

Figure 6 illustrates genotyping of the –28C/G and –403G/A RANTES Promoter polymorphisms. Genotypes and restriction enzymes used are indicated on top of the figure, while sizes of diagnostic fragments and molecular weight (MW) markers (100 bp DNA ladder, Life Technologies) are shown in the left and right margins for the –403 and –28 sites respectively. Fragments less than 50 bp are not well visualised in these gels.

Figure 7 shows RANTES promoter genotype frequencies in different clinical categories. Frequencies (%) of Genotypes 1 and 4 (-403G/G, -28C/C and -403G/A, -28C/C left and right panels respectively) in random blood donors (RBD) versus exposed, uninfected (EU), and HIV infected (HIV+) individuals from the MACS. All samples were from Caucasian individuals who lacked CCR5Δ32.

Figure 8 shows RANTES promoter genotype and progression to AIDS-1993. Kaplan-Meier survival curves for MACS seroconverters with Genotypes 1 and 2 (n=136, -403G/A, -28C/C and -403A/A, -28C/C, solid line) versus Genotype 4 (n=185, -403G/G, -28C/C, dashed line) for the endpoint of AIDS-1993 as defined by the U.S. Centres for Disease Control. Median times to the endpoint were 5.4 years versus 6.7 years respectively with a relative hazard of 0.72 and a p value of 0.03.

EXAMPLES

1. -400 Polymorphism (Nelson sequence)

A single base polymorphism at position –400 (relative to the transcription start site in the Nelson et al sequence) has been characterised in the promoter region of the human RANTES gene. RANTES gene promoter polymorphisms were examined by amplifying 969 bp upstream of the transcription site, followed by SSCP and direct sequencing of PCR products.

In patients with a wild type RANTES gene the base at potion –400 relative to the transcription start site (in the Nelson sequence) is Guanine (the G allele). In patients with a polymorphic RANTES promoter sequence the base at position –400 relative to the transcription start site (in the Nelson sequence) may be Adenine (the A allele).

The presence of the RANTES promoter –400 polymorphism (Nelson sequence) (the A allele) was determined in certain groups, to show the link between the presence of the polymorphism and two conditions associated with abnormal RANTES gene expression, that is asthma and protection from HIV.

Subjects:

Asthma group	Number
Atopic non asthmatic	70
Atopic asthmatics	40
Non atopic non asthmatics	23

HIV group	Number
Haemophiliacs HIV +ve	91
Haemophiliacs HIV –ve	13
Partners of HIV patients	21

Method

A G→A transition polymorphism at position –400 in the human RANTES promoter relative to the transcription site (of the Nelson sequence) was characterised. Neither the wild type nor the mutant allele abolishes or introduces a restriction enzyme site. To create an enzyme site that can be used to differentiate between wild type/polymorphic alleles, a single base change was introduced at position –396 relative to the transcription site of the Nelson sequence (C→G, in the reverse sequence). In the presence of the wild type G allele the *Mae* III enzyme cuts the PCR product giving two bands of 112 and 23 bp and in the presence of the polymorphic A allele the enzyme does not cut the PCR product (135 bp).

Screening

The following primers were designed based on the genomic sequence of RANTES based on the Nelson sequence.

Forward	5' GCC TCA ATT TAC AGT GTG 3' (SEQ ID No. 1)
Reverse	5' TGC TTA TTC ATT ACA GAT GTT3' (SEQ ID No. 2)

The position of the forward primer starts at –513 and the position of the reverse primer is at position –378 of the Nelson sequence. The PCR product length generated is 135bp. A G based (underlined) at the 3' of the reverse primer was mutated from a C to introduce an enzyme site for *Mae* III.

PCR Conditions

The PCR reactions were carried out in 25 µl reactions containing 1 x (NH4 buffer, Bioline), 1.5 mM Mg_2, 0.2 mM dNTPs (Bioline), 6.3 pmol of each primer and 1U Taq polymerase (Bioline) and 1 mM Betaine (Sigma).

PCR Conditions

PCR cycles were as follows:

1 cycle at 95°C for 2min

35 cycles each of 95°C for 40 sec, 50°C for 40 sec and 72°C for 40 sec.

A final extension step was carried out at 72°C for 5 min.

Restriction Enzyme Digestion

The digestion reaction was carried out in 15µl reactions comprising 5 units *MaeIII* enzyme, 7.5 µl reaction buffer and 5.5 µl PCR products, incubated overnight at 55°C. The products of the restriction enzyme digestion were visualised on 3% agarose gel stained with ethidium bromide.

Results

The digested products as visualised on 3% agarose stained with ethidium bromide (see Fig. 4) showed that individuals homozygous for the wild type G allele yield two bands of 112 and 23 bp. The homozygous polymorphic A allele yields one uncut band (135 bp).

Allele Frequency

The following frequencies were observed in a total of 90 healthy UK Caucasoids:

Allele	**Frequency**
A	0.161
G	0.839

The observed heterozygosity was 0.211.

As shown in the attached table (Figure 5) in the asthmatic group the heterozygous AA polymorphic frequency was found to be increased in atopic asthmatics and asthmatics. Furthermore, in the group exposed to HIV infection, there was a significant increase in the AA frequency in exposed uninfected individuals only.

Discussion

The AA polymorphism seems to be associated with a tendency to asthma and protection from HIV infection. In both conditions high levels of RANTES may be implicated.

2. -28 Polymorphism

A single base polymorphism at position –28 has been characterised in the promoter region of the human RANTES gene.

Method

A C→G transversion polymorphism at position –28 in the human RANTES promoter relative to the transcription site (of both the Nelson and Lui sequences) was characterised. The G allele abolishes a *Mnl I* restriction enzyme site.

In the presence of the wild type C allele the *Mnl I* enzyme cuts the PCR product giving three bands of 126, 27 and 20 bp. In the presence of the polymorphic G allele only two bands are detected, 146 and 27 bp.

Screening

The following primers were designed based on the genomic sequence of RANTES based on the Nelson sequence.

Forward primer: 5' ACA GAG ACT CGA ATT TCC GGA 3' (SEQ ID No. 3)

Reverse primer: 5' CCA CGT GCT GTC TTG ATC CTC 3' (SEQ ID No. 4)

The position of the forward primer starts at –145 and the position of the reverse primer is at position +28 of the Nelson sequence. The PCR product length generated is 173bp.

PCR Conditions

A total of 100 ng genomic DNA was amplified in a 25 µl final volume PCR reaction containing containing 1 x (NH4 buffer, Bioline), 1.5 mM Mg_2, 0.2 mM dNTPs (Bioline), 6.3 pmol of each primer and 1U Taq polymerase (Bioline) and 1 mM Betaine (Sigma).

PCR Conditions

PCR cycles were as follows:

1 cycle at 95°C for 2min

35 cycles each of 95°C for 40 sec, 50°C for 40 sec and 72°C for 40 sec.

A final extension step was carried out at 72°C for 5 min.

Restriction Enzyme Digestion

The digestion reaction was carried out in 15µl reactions comprising 3 units *Mnl I* enzyme and 5 µl PCR products, incubated overnight at 37°C. The products of the restriction enzyme digestion were visualised on 4% Nuseive agarose gel stained with ethidium bromide.

Results

The digested products as visualised on 4% Nuseive agarose gel stained with ethidium bromide showed that individuals homozygous for the wild type C allele yield three bands of 126, 27 and 20 bp. The homozygous polymorphic G allele yields two bands of 146 and 27 bp.

Allele Frequency

The following frequencies were observed in a total of 90 healthy UK Caucasoids:

Allele	Frequency
G	0.04.4
C	0.95.6

The observed heterozygosity was 0.089.

3. –403 POLYMORPHISM (LIU SEQUENCE) AND PROTECTION FROM HIV NFECTION

Methods In all of the following examples the RANTES gene sequence is enumerated according to Lui *et al.*

The dimorphic single nucleotide polymorphisms (SNPs) designated –403G/A (Lui sequence) and –28C/G, in the RANTES promoter were analysed. Genotype frequencies were compared between normal blood donors and two groups of participants in the Multicenter AIDS Cohort Study (MACS) of homosexual men: 1) HIV+ participants and 2) highly exposed, uninfected participants (EU). Kaplan-Meier analysis was used to analyse the association of genotype with rate of progression to AIDS in MACS seroconverters.

Findings. We found reciprocal distortion of expected frequencies of genotypes –403G/A, -28C/C and -403G/G, -28C/C in EU versus HIV-negative individuals (Odds Ratio [OR]=1.68, p=0.021), which increased when individuals possessing CCR5Δ32 were omitted from consideration (OR=2.06, p=0.011). Specifically, -403G/A was enriched in the HIV+ population and –403G/G was enriched in the EU population. In contrast, seroconverters lacking CCR5Δ32 progressed significantly faster to AIDS (1993 definition) if they had genotype –403G/G, -28C/C versus the combined group of CCR5Δ32 negative seroconverters with –403G/A, -28C/C or –403A/A, -28C/C (median time to AIDS=5.4 versus 6.7 yrs; relative hazard [RH]=1.39; p=0.03).

Interpretation These results provide the first genetic evidence that risk of HIV infection is modulated by chemokine ligands, and specifically identify RANTES promoter allele –403G as protective relative to –403A (Lui sequence) for initial HIV infection in homosexual men. We propose that promoter polymorphism transcriptionally regulates RANTES production and blockade of CCR5 usage by HIV. The opposite effect noted on progression may result from paradoxical stimulation of HIV replication in infected cells, as noted in recent studies.

Abbreviations OR, odds ratio; RH, relative hazard; CDC, United States Centres for Disease Control; LTNP, long term non-progressor: EU, exposed, uninfected; MACS, Multicenter AIDS Cohort Study; bp, basepair; PBMC, peripheral blood mononuclear cell; SSCP, single stranded conformation polymorphism; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; RBD, random blood donor; tsp, transcription start point; UTR, untranslated region; EMBL, European Molecular Biology Laboratory

Methods

Study Participants

Participants in the study were anonymous blood donors from the Warren Grant Magnuson Clinical Centre at the National Institutes of Health and the American Red Cross, anonymous blood donors from West Africa collected for onchocerciasis epidemiology research,[36] and participants in the Multicenter AIDS Cohort Study (MACS) studied under an approved protocol.[37,38] Blood donors in all cohorts were classified into self-described racial groups. Random donor samples were received either as peripheral blood or PCR-ready DNA. MACS samples were acquired as PBMCs from which genomic DNA was purified.[23] All samples which yielded PCR product were genotyped by the method described below and used in the presented data. 98% of MACS samples were informative.

The MACS study began in 1984-85 and now includes over 5000 men from four centres in the U.S, who have had biannual follow-up. The EU cohort (n=123) used in this paper was primarily composed (n=95) of those MACS participants who were in the highest 10% of risk for HIV infection as defined by the number of anal receptive intercourse partners in the 2.5 years prior to their second MACS visit.[23,39] An additional 28 men were included from whom HIV-1 was isolated but who subsequently have remained seronegative.[40] This guarantees the inclusion of extremely highly exposed individuals in this investigation.

Other MACS participants included in this study were HIV+ (n=672). Of these, 506 were seroconverters, defined as subjects who were HIV-1 seronegative at the time of enrolment in the study who subsequently seroconverted. However, only individuals with less than nine months elapsed time between testing as HIV negative and positive were included in the seroconverter group analysis (n=406) to ensure a uniform study population. Time of infection was chosen as the midpoint between the date of the first HIV seropositive visit and the date of the last negative HIV-1 blood sample. When analysing progression effects, a cut-off date of 1/1/96 was used to avoid possible confounding effects of highly active anti-retroviral therapy. The remaining 266 HIV+ subjects under study did not meet the seroconverter definition for the progression analysis (i.e. they entered the study seropositive or had greater than nine months time elapsed between their last HIV seronegative and first HIV seropositive dates). Demographic characteristics for EU and HIV+ cohorts were similar (Table 1). CD4 count decline was analysed using linear regression to calculate the CD4 slope from the date of the third seropositive visit until the participant initiated potent anti-retroviral therapy. Since, at least two CD4 counts during this period were needed to calculate the slope, this eliminated 45 of the 406 seroconverters from this analysis.

Genotyping

Genotyping of the two sites was carried out using identical PCR conditions as described in our co-pending application with minor modifications. DNA samples

were amplified in 30 µl reactions containing 1X NH_4 buffer (BioLine, Reno, NV), 2 mM $MgCl_2$, 0.2 mM dNTPs (Life Technologies, Baltimore, MD), 1 mM Betaine (Sigma, St. Louis, MO), 250 µM of each primer, and 1.2U of Taq (Life Technologies, Baltimore, MD). The thermocycler settings were as follows: a hot start of 95°C for 2 min; followed by 35 cycles each of 95°C for 40 sec, 50°C for 40 sec, and 72°C for 40 sec; and ending with a final extension of 72°C for 5 minutes.

In order to perform the genotyping for –403-G/A we first used the conditions above and the primer set comprising SEQ ID NO 1 and SEQ ID NO 2 as described above.

This results in a 136 bp product amplified from position –516 through –381. We then performed a *Mae* III restriction enzyme digestion in a 20 µL volume in the supplied buffer with 1U of enzyme (Roche Molecular Biochemicals, Indianapolis, IN) and 5 µL of PCR product cut at 55°C overnight. Since neither of the alleles at the –403 site creates or destroys a natural restriction enzyme site, we introduced a deliberate mismatch in the –403 reverse primer (underlined above) at the –399 position. This creates a *Mae* III site (/GTNAC) if there is a G in the –403 position which will cleave the 136 bp fragment into pieces of 113 and 23 bp lengths. The –403A allele remains undigested (Figure 1).

Genotyping of the –28G/C polymorphism was performed by using the conditions above and the primer set comprising SQ ID NO 3 and SEQ ID NO 4 as described above.

This creates a 187 base pair product amplified from position –147 through +40. We then used 3U of *Mnl* I enzyme (New England BioLabs, Beverly, MA) in the recommended buffer to cut 5 µL of PCR product in a 20 µL reaction overnight at 37°C. *Mnl* I (CCTC (7/6)) cuts the product at 3 nonpolymorphic sites and 1 polymorphic site (-31 through –28 in the RANTES promoter). The G allele at –28 eliminates one site allowing the digestion to produce only four fragments (134 bp, 27

bp, and two 13 bp fragments), while the C allele at –28 results in four cutting sites which yield five fragments of 114 bp, 27 bp, 20 bp, and two 13 bp length (Figure 7).

All digestion products were visualised on 4% NuSieve GTG agarose gel stained with GelStar (FMC Bioproducts, Rockland, ME) per manufacturer's recommendations.

Statistics

The differences in genotypic and haplotypic frequencies for the RANTES promoter were examined for significance using the Pearson chi square test. HIV progression was analysed using Kaplan-Meier estimates of time to AIDS-1993 (as determined by the U.S. Centres for Disease Control). Determination of significance of survival curves was based on Cox proportional-hazards models. Effects on CD4 count decline were analysed using a Wilcoxon Rank Sum test.

Results

Using SSCP and genomic DNA, we recently identified two dimorphic SNPs at sites 28 bp and 403 bp 5' of the transcription start point (tsp), as defined by Lui et al, by screening a 944 bp region of the RANTES promoter region from 40 individuals.[42] We then developed a rapid genotyping method based on PCR-RFLP.[43,44]

Here we analysed the distribution of genotypes in different racial and clinical groups. The two SNPs are in linkage disequilibrium such that only three haplotypes are seen rather than the four that are theoretically possible. Specifically, we did not observe any individuals with a –403G/–28G haplotype. This is most likely due to a lack of cross-over events because of the close physical proximity of the two polymorphisms and the way that these two SNPs have arisen in human evolution. Alternatively, the –403G/–28G haplotype could markedly diminish reproductive fitness.

Haplotype –403G/-28C was observed at high frequency in all racial groups tested (47-81%). Haplotype –403A/-28C was found at somewhat lower frequency and with

much greater variability among racial groups, e. g. a 2-3 fold difference between Caucasians and Blacks. Haplotype –403A/-28G was almost never observed in Blacks and Hispanics and was relatively uncommon in Caucasians (4%) (Table 2). Allelic and genotypic frequencies of the –28 and –403 polymorphisms were in Hardy-Weinberg equilibrium within each racial group of random donors, indicating a lack of historical selective pressure on either of the two distinct polymorphic sites.

We next analysed the haplotype and genotype frequencies in EU and HIV+ subjects from the MACS. Previously we reported that homozygous CCR5Δ32 individuals were much more common in EU versus HIV+ subjects from the same cohort (5% vs. 1%).[23] We now found that the haplotype –403A/-28C was more common in HIV+ versus EU MACS participants (19.6% vs.14.2%). Conversely, haplotype –403G/-28C was more common in EU versus HIV+ MACS participants (84.1% vs.77.8%; p=0.041, Tables 3 and 4). Since, CCR5Δ32 homozygosity is known to diminish the probability of HIV transmission and CCR5Δ32 heterozygotes are known to have diminished levels of CCR5 on the cell surface, we reasoned that any effects of RANTES promoter polymorphism on transmission might be more evident if we omitted from consideration all individuals that had a CCR5Δ32 allele.[23] As shown in Tables 3 and 4, this caused an increase in the difference in frequency for both haplotypes in HIV+ versus EU groups (haplotype –403A/-28C: 20.8% in HIV+ vs. 12.4% in EU; and haplotype –403G/-28C: 86.0% in EU vs. 76.8% in HIV+; p=0.007). Because of the variation in allele frequencies in different racial groups, we next limited the analysis to Caucasians who lack CCR5Δ32. Despite the lowered numbers of individuals available for comparison, the difference in haplotype frequencies persisted in HIV+ versus EU comparisons and remained significant (haplotype –403A/-28C: 17.5% in HIV+ vs. 10.8% in EU; and haplotype –403G/-28C: 88.0% in EU vs. 79.8% in HIV+, p=0.03). Thus racial bias in the comparison groups could not explain the results. Differences in genotype frequencies in HIV+ versus EU groups followed the same pattern and magnitude as for the haplotype analysis (Tables 3 and 4, Figure 8).

In Caucasian random blood donors who lacked CCR5Δ32, frequencies for each of these two haplotypes were intermediate between those of the EU and HIV+ groups. This is consistent with a selective pressure acting to enrich the -403G/-28C haplotype in EU populations while depleting it in HIV+ populations, and vice versa for the -403A/-28C haplotype (Figure 8). Collectively, these data strongly suggest that the –403G allele may confer protection relative to the –403A allele for homosexual transmission of HIV. Since less than 3% of the population studied had the –403A/-28G haplotype and the –403G/-28G haplotype wasn't seen, we were limited in our ability to analyse the effects of these haplotypes on HIV transmission or progression.

The effect of the –403A allele on HIV transmission seemed to be dominant since individuals of genotype –403G/A, -28C/C were more common in the HIV+ group than in the EU group (29.9% vs.21.1%) and the –403G/G,-28C/C genotype was more common in the EU group than the HIV+ group (72.4% vs. 60.6%, p=0.027). We used this comparison to eliminate any effect of the –28 allele and because other genotypes are rare in this cohort.

Finally, we tested the effects of RANTES promoter polymorphism on HIV progression using seroconverters from the MACS cohort. These individuals have a very precisely defined date of infection because they entered the study HIV seronegative, were tested every six months, and were subsequently observed to be HIV seropositive. Therefore, these individuals are an ideal cohort to study the effects of a genotype on progression rate. MACS seroconverters possessing a –403A allele (–403G/A,-28C/C or –403A/A, -28C/C genotypes) progressed more slowly to AIDS than those lacking this allele (–403G/G,-28C/C genotype), although the difference did not reach statistical significance (median time to AIDS 1993: 7.1 versus 5.6 years; RH 0.79; p=0.078) When seroconverters possessing a CCR5Δ32 allele were removed from the analysis , the difference reached statistical significance (median time to AIDS-1993 6.7 versus 5.4 years; RH-0.72, p=0.03).(Figure 3).

Discussion

Our results show that RANTES promoter polymorphism –403 G/A is a risk factor for HIV transmission and progression in homosexual men, and provide the first genetic evidence in support of the hypothesis that variation in HIV coreceptor ligands can modulate risk of HIV transmission. SDF1-3'A, a previously described polymorphism which affects the 3'-UTR of the mRNA for the CXCR4 ligand SDF-1, is a risk factor for HIV progression but not transmission.[30] Instead, genetic restriction of HIV transmission has been clearly shown for the chemokine receptor CCR5 by disease association analysis of the CCR5Δ32 mutation, which inactivates CCR5.[19-23] Although RANTES promoter polymorphism exerts a weaker effect than homozygous CCR5Δ32 on the risk of HIV transmission, it is important to appreciate that the effect achieved statistical significance within a single cohort, which has typically not been the case for effects of CCR5Δ32, SDF1-3'A and CCR2-64I on the risk of disease progression in seroconvertor cohorts,[19-23, 27-31] including the MACS (D McDermott, P Zimmerman, C Kleeberger and P Murphy, unpublished results).

Since RANTES can block CCR5's HIV coreceptor activity *in vitro*, it is reasonable to postulate that the effects of RANTES promoter polymorphism are mediated directly, through modulation of RANTES transcription and protein production, resulting in differential blockade of HIV access to CCR5. This hypothesis would be consistent with other experiments that have shown that EU individuals have cell types that tend to secrete more RANTES.[32,33] The results suggest a potential mechanism to explain HIV resistance in a portion of the 95% of MACS EU individuals who are not CCR5Δ32 homozygotes.[23] However, it is important to realise that any protection of a certain genotype is relative and not absolute. Even among CCR5Δ32 homozygotes, who have no functional CCR5 there have been a growing number of HIV infected individuals identified.[45,46]

Not only may the effects of a genetic risk factor in disease vary in penetrance depending on both environmental and host factors, they may also conceivably have

opposite effects on different aspects of pathogenesis. Thus, in our study we found that, compared to the –403A allele, the –403G allele was associated with decreased susceptibility to HIV transmission but increased rate of HIV progression in HIV+ individuals. The mechanistic basis for this may relate to the ability of RANTES to block initial HIV entry during transmission, which appears to be predominantly through CCR5, while stimulating HIV replication in already infected cells, perhaps through effects on the activity of CXCR4, other coreceptors, or even CCR5 itself, as has been noted in previous studies. [13-15,17] Understanding this is important since any therapeutic or preventative intervention involving chemokine analogues must define whether the agent is acting as an agonist or antagonist of HIV infection *in vivo*.

Both the –403 and –28 sites include recognition sequences for known transcription factors according to an electronic search using the Transfac version 3.2 program from the University of Pennsylvania available on the world wide web at http://agave.humgen.upenn.edu/tess/index.html.[47] In the case of –403A, a potential site for GATA-1 or GATA-2 exists which is no longer reported by the program when the –403G allele is present. For –28C a potential c-Myc site is present which is removed by –28G which instead creates a potential glucocorticoid receptor site. In addition, the –28 site is adjacent to the TATA box which is critical to promoter function. Future work will be necessary to determine whether these potential sites are used by these or other transcription factors to regulate RANTES levels in relevant cell types.

Liu, *et al.* reported the same two polymorphisms reported here, and found that the –403A/-28G haplotype was associated with slower CD4 decline, but did not detect any association with altered risk of HIV transmission.[48] Their progression analysis did not look at clinical endpoints. Our study had only 17 seroconverters with the –403A/-28G haplotype because the MACS participants are mostly Caucasian and we may have been underpowered to see a small or modest effect on HIV progression rate. However, in agreement with Liu et al., we did see a reduction in the CD4 count

decline rate in the few individuals who possessed a –403A/–28G containing haplotype versus all others that could be analysed, although it did not quite reach statistical significance (n=15, median of –37.28/year vs. –66.76/year, p=0.08).

There are two important differences between our study and Liu *et al.* that must be considered in interpreting the different results regarding transmission: first, Liu *et al.* studied primarily Japanese haemophiliacs whereas we focused on Caucasian homosexuals; and second, their HIV uninfected group included only 50 individuals with possible exposure to HIV whereas ours included 123 individuals with clearly quantified high exposure. Despite the differences, however, it is important to note that both studies support the idea that RANTES promoter polymorphism modulates risk in HIV disease. Differences in risk based on race, type of exposure, and cohort composition and definition have also been reported previously for the CCR5Δ32, SDF1-3'A and CCR2-64I polymorphisms.[19-23,27-31]

It is interesting to note the much higher frequency of the –403A allele in Black African than Caucasian and Asian individuals. This suggests that there may exist or have existed a strong selective pressure in favour of this allele. This may have involved differential susceptibility to ancestral plagues. A compelling and related example of this is the Duffy antigen receptor for chemokines, which appears to cause genetic resistance to infection with the malaria-causing protozoan *Plasmodium vivax* through polymorphism in the promoter.[49-51]

Our results provide evidence that RANTES levels are important to the transmission and progression of HIV disease. They may also have implications for diseases other than HIV. RANTES is believed to be involved in diverse inflammatory disorders, such as rheumatoid arthritis[52-54], asthma[55], glomerulonephritis[56], endometriosis[57], inflammatory bowel disease[58], and multiple sclerosis.[59] In these conditions, RANTES is felt to play a pro-inflammatory role. Further study of natural variation in

chemokine gene structure and function may identify therapeutic targets for these conditions, in addition to HIV.

4. –403 POLYMORPHISM AND ASTHMA

The example described earlier in relation to the –400 and –28 polymorphisms (as compared to the Nelson sequence) and asthma was repeated and the results of the repeat experiments are shown below in terms of the number and percentage of individuals carrying the mutant alleles for RANTES –28 and –403 polymorphisms (as compared to the Lui sequence).

	-28 G	-403 A
Non atopic / non asthmatic	2/21	2/21
Atopic /non asthmatic	0/4	3/4
Atopic / asthmatic	3/22	7/22

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Table 1: **Baseline characteristics of the MACS participants used in this study separated by clinical category.** All participants are men who have sex with men.

Clinical Category	Exposed, Uninfected	HIV Infected
Number of Participants	(n=123)	(n=672)
Median Age at Entry	34.2 years	31.2 years
Caucasian, Non-Hispanic	89.4%	87.5%
Black, Non-Hispanic	1.6%	6.9%
Hispanic	8.1%	5.2%
Median number of partners in the 2.5 years prior to visit 2	62	20

Table 2: **RANTES promoter genotype and haplotype frequencies (%) in blood donors of different races/ethnicities**

		RANTES Promoter Sites		Caucasians	North Am. Blacks	W.African Blacks	North Am. Hispanics	North Am. Asians
		-403	-28	n=200	n=151	n=173	N=53	n=61
Genotype	1	G/G	C/C	67.0	38.1	20.8	56.6	55.7
	2	A/A	C/C	2.5	9.5	25.4	1.9	4.9
	3	A/A	G/G	0.5	0.0	0.0	0.0	0.0
	4	G/A	C/C	23.0	52.4	53.2	37.7	27.9
	5	G/A	C/G	5.5	0.0	0.0	1.9	6.6
	6	A/A	C/G	1.5	0.0	0.6	1.9	4.9
Haplotype								
	I	G	C	81.3	64.3	47.4	76.4	73.0
	II	A	C	14.8	35.7	52.3	21.7	21.3
	III	A	G	4.0	0.0	0.3	1.9	5.7

Table 3: RANTES promoter genotype and haplotype frequencies (%) in EU vs. HIV+ individuals from the MACS cohort. Groups were compared with and without individuals who have a CCR5Δ32 allele and also to Caucasian RBD individuals who lack CCR5Δ32.

		RANTES Promoter Sites		All Samples		All Samples without CCR5Δ32		Caucasians only without CCR5Δ32		
				EU	HIV+	EU	HIV+	EU	RBD	HIV+
		-403	-28	n=123	n=672	n=89	n=542	n=79	n=151	n=461
Genotype	1	G/G	C/C	72.4	60.6	75.3	59.2	78.5	66.9	62.9
	2	A/A	C/C	3.3	4.5	2.3	5.2	1.3	2.6	2.6
	3	A/A	G/G	0.0	0.2	0.0	0.2	0.0	0.0	0.2
	4	G/A	C/C	21.1	29.9	19.1	31.0	17.7	23.8	29.3
	5	G/A	C/G	2.4	4.6	2.3	4.1	1.3	5.3	4.6
	6	A/A	C/G	0.8	0.3	1.1	0.4	1.3	1.3	0.4
Haplotype										
	I	G	C	84.1	77.8	86.0	76.8	88.0	81.5	79.8
	II	A	C	14.2	19.6	12.4	20.8	10.8	15.2	17.5
	III	A	G	1.6	2.6	1.7	2.4	1.3	3.3	2.7

EU (exposed, uninfected) and HIV+ (HIV infected) individuals from the MACS cohort; RBD, random blood donor

Table 4: **Pearson chi square tests of significance of data from Table 3.**

Comparisons (degrees of freedom)	**All samples**		**All samples without CCR5Δ32**		**Caucasians only without CCR5Δ32**	
	Chi square value	p value	Chi square value	p value	Chi square value	p value
Comparison of all three haplotypes (2)	5.03	0.081	7.60	0.022	6.49	0.052
Haplotype I vs. Haplotypes II (1)	4.17	0.041	7.22	0.007	4.70	0.030
Genotype 1 vs. Genotypes 2 and 4 (1)	5.37	0.021	8.04	0.005	6.08	0.014
Genotype 1 vs. Genotype 4 (1)	4.90	0.027	6.54	0.011	5.49	0.019
-403 G/G vs. G/A vs. A/A (ignoring -28 genotypes) (2)	6.27	0.033	8.32	0.016	7.32	0.026